



DECLARATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Noel John de Souza et al.
Serial No: 10/033,366
Filed: December 27, 2001
For: Treatment of Immune System-Modulated Disorders

Group:
Examiner: Flood, Michele C

Attorney Docket : U013784-9

Assistant Commissioner for Patents
Washington,
D.C. 20231

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I, Noel John de Souza, M.Sc. Ph.D., having 41 years of Drug Discovery Research experience holding currently the position of Director – R&D at Wockhardt Research Centre Aurangabad, a citizen of India declare

That I have read and understood the specification of US patent application 10/033,366

That the following method was used for preparation of aqueous solutions of 3 samples of *Tinospora cordifolia* Dry Water Extract provided during January – March 2003 by supplier to Wockhardt Limited.

An accurately weighed amount (x) of about 0.75 gm of the provided *Tinospora cordifolia* Dry Water Extract sample was transferred to a flask. To it was added 1.4 ml of water, the mixture sonicated, heated to about 80 °C for about 1 hr and then allowed to come to room temperature (25°C). The entire content of the flask was transferred to a centrifuge tube and centrifuged for 10 mins at 4600 rpm. The supernatant layer (1.2 ml) was transferred to an Eppendorf tube and submitted for checking of its antibacterial activity. The concentration of the extract solution was determined by transferring the residue in the centrifuge tube to a petri-dish, keeping in an oven at 100 degrees centigrade for drying to constant weight (y) and calculating the concentration from the formula $(x - y) \div 1.2$

Sample A (# 2301121) :	Conc. of solution : 0.471gm/ml
Sample B (# 2303273) :	Conc. of solution : 0.433gm/ml
Sample C (# 2303352) :	Conc. of solution : 0.437gm/ml

That the following method was used for testing of aqueous solution of *Tinospora cordifolia* Dry Water Extract for antibacterial activity:

Method : Quantitative Agar Diffusion Assay
 Media : Mueller Hinton Agar (MHA) (Difco, USA)
 Organisms : *E. coli* ATCC 25922, ESS (*E. coli* - super sensitive), *S. aureus* ATCC 25923, *S. epidermidis* 108,
 Inoculum : 1×10^5 to 5×10^5 CFU/ml

Procedure:

- 160 ml of molten sterilized MHA with the specified overnight grown inoculum in Tryptic Soya Broth was poured on to the bioassay dish (Nalge Nunc, USA/Nunc AS, Denmark, 243 X 243 X 18 mm) with final inoculum of 10^5 to 5×10^5 CFU/ml. Poured plates were placed on a very flat surface and were allowed to solidify.
- Plates were transferred to refrigerator ($4 - 8^\circ \text{C}$) for the agar to set firmly. 6-mm diameter wells were punched with the help of a borer at a distance of approximately 5-6 cm.
- Solutions with the following concentrations were diluted 1:1 serially in water up to two dilutions.
- All dilutions were tested by adding 50 ul of each sample in the wells.
- Plates were incubated in ambient air for 18-24 h at 37 degree centigrade.
- Zone diameters were measured with a measuring scale.

The results are shown in the following table:

Antibacterial Activity of Aqueous Solution of *Tinospora cordifolia* Dry Water Extract

Organisms	Zone of Inhibition (mm)			
	Tinospora Cordifolia Extract (Aq. Soln.)			Berberine
	Sample A	Sample B	Sample C	
	(Conc. mg/ml)			
	471	433	437	2
1) E.coli ATCC 25922	15	0	0	0
2) E.coli supersensitive (ESS)	20	10	10	14
3) S.aureus ATCC 25923	15	0	0	10
4) S. epidermidis 108	10	0	0	0

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Signed this 4th day of June 2003



Noel John de Souza, Ph.D.
Director - R&D



ANNEXURE-1

Tinospora cordifolia - PMN and LC-MS data

Sr. No.	Batch No.	PMN % (Limit NLT 120%)	Peak Area (m/z 341)	Peak Area (m/z 481)	Total Peak Area of two peaks	% of Peak 1 (Limit NMT 85%)	% of Peak 2 (Limit NLT 35%)	Remarks	Case
1	803081	138.7	12802	34842	47644	26.87	73.13	Passes	4
2	804132	132.0	8924	33852	42776	20.86	79.14	Passes	4
3	804108	137.4	14310	20728	35038	40.84	59.16	Passes	4
4	808253	140.0	14418	13896	28314	50.92	49.08	Passes	4
5	P004612	138.1	35487	57486	92973	38.17	61.83	Passes	4
6	P003307	132.8	53575	45600	99175	54.02	45.98	Passes	4
7	P003319	127.4	48301	42140	88441	52.35	47.65	Falls	1
8	ACD/RAD/1098	117.0	71251	1557	72808	97.86	2.14	Falls	1
9	804147	110.0	17003	4512	21515	79.03	20.97	Falls	1
10	808276	118.0	88159	0	88159	100	0	Falls	1
11	804148	112.0	8220	4410	12630	65.08	34.92	Passes	4
12	2106805	134.3	22891	39958	62849	38.22	63.78	Passes	4
13	2103472	133.3	28446	23051	49497	53.43	48.57	Falls	2
14	2103390	135.2	52487	12760	65247	80.44	19.56	Falls	2
15	2103389	128.7	39378	12282	51661	76.23	23.77	Falls	2
16	121802	131.7	44924	11044	55968	80.27	19.73	Falls	4
17	121801	133.6	28319	27540	55859	48.87	51.13	Passes	4

Case	LC-MS-SIR	PMN activity	Remark
1	Not OK	Not OK	Falls
2	Not OK	OK	Falls
3	OK	Not OK	Falls
4	OK	OK	Passes